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A high throughput membrane BIO-PCR technique for ultra-sensitive detection of *Pseudomonas syringae* pv. *phaseolicola*

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Molecular-based methods such as PCR have greatly improved detection of bacteria in environmental samples. However, the sensitivity of PCR is not high when compared to agar plating assays, and inhibitors from plants are often a problem. Pre-enriching bacteria on agar media (BIO-PCR) can increase the sensitivity of PCR by more than 100% and reduce the effects of inhibitors. To further increase the sensitivity and also reduce the labour needed for BIO-PCR, a high throughput 96-well membrane BIO-PCR technique is described for ultra-sensitive detection of *Pseudomonas syringae* pv. *phaseolicola* (PSP) (syn. *P. phaseolicola*) in washings of seeds and leaves of *Phaseolus vulgaris*, using available classical PCR primers and newly designed real-time primers and probe. The primers and probe, designed from a tox-argK chromosomal cluster of the PSP-specific phaseolotoxin gene, were confirmed to be specific to PSP. Samples (1·2 mL) were filtered under vacuum in 96-well membrane plates. After incubating on soft agar medium for 48–52 h, each well is washed with 200 μL of sterile water and used immediately for nested (two-step) PCR or real-time PCR or stored at –20°C. Results of assaying spiked seed washings showed that classical PCR was unable to detect PSP at mean concentrations of 40 colony forming units (cfu) mL^{-1}. BIO-PCR detected PSP in five out of six samples at 40 mean cfu mL^{-1} but none at mean concentrations of 4·2 and 0·4 mean cfu mL^{-1}. In contrast, membrane BIO-PCR detected the bacterium in all six samples tested containing as few as 0·4 mean cfu mL^{-1}. The sensitivity of detection from leaf washings was lower but the results were similar, classical and BIO-PCR were negative from all three levels of inoculum while membrane BIO-PCR detected three out of three samples at 80 mean cfu mL^{-1} and one out of three at 40 mean cfu mL^{-1}.

Keywords: nested PCR, *Phaseolus vulgaris*, real-time PCR, vacuum filtration

Introduction

Detecting bacterial pathogens in seeds and other plant materials such as symptomless leaves is often difficult because the population of the target organism is often less than other bacteria. Classical isolation methods can be very sensitive but not when samples contain few numbers of the target organism and large numbers of other bacteria (Schaad, 1989). Serological techniques are rapid and inexpensive but are generally insensitive and lack specificity (Hampton *et al*., 1990). With recent advances in PCR technology, considerable progress is being made in the detection of small numbers of microorganisms even in the presence of numerous other organisms. The sensitivity of classical PCR can be increased with the use of a second round of PCR using nested primers (Schaad *et al*., 1995). However, PCR has several limiting factors including PCR inhibitors often present in natural plant samples (Rossen *et al*., 1992; Prosen *et al*., 1993; Schaad *et al*., 1999; Weller *et al*., 2000), and relatively low sensitivity due to extremely small sample size requirements. Sensitivity can be further increased with techniques such as immunomagnetic separation (IMS-PCR) (Skjerve & Olsvik, 1991; Fratamico *et al*., 1992; van de Wolf *et al*., 2000) or enrichment PCR (BIO-PCR) (Schaad *et al*., 1995; Ito *et al*., 1998; Weller *et al*., 2000). Both techniques can reduce the effect of PCR inhibitors, but BIO-PCR has the added advantage of including the recovery of a viable culture of

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the target organism. This technique combines biological amplification on common or semiselective media (agar or liquid) with direct PCR; DNA extraction is not required, nor is a centrifugation step needed for concentrating the target. Typically, samples of 0·1 mL are plated onto each of 6–8 agar medium plates and the resulting cell growth removed from half of the plates by washing with water after 24–72 h incubation, depending upon the growth rate of the target organism. The pooled samples containing cells of the target organism are used immediately for PCR or stored at –20°C for use later. BIO-PCR has a major advantage over classical agar plating techniques. Whereas large numbers of other organisms often overgrow the target organism on agar media before the organism can be recognized, this does not happen in BIO-PCR because colonies of the target organism are harvested when they are just visible and colonies of saprophytes are relatively small. The sensitivity of BIO-PCR (normally 5–10 mean colony forming units (cfu) mL^{-1} of sample) can be 10 to 100-fold more sensitive than classical PCR (Schaad *et al*., 1995; Ito *et al*., 1998; Schaad & Frederick, 2002). Such sensitivity, however, is still not good enough for detecting regulated and/or quarantined plant pathogenic bacteria which are often present in very small numbers in asymptomatic tissues (Webster *et al*., 1983; Ozakman & Schaad, 2003).

In this study *Pseudomonas syringae* pv. *phaseolicola* (PSP) (syn. *P. phaseolicola*) was chosen as a model pathogen because the organism is seed-borne and there is a zero tolerance for PSP infection in bean seed lots (Webster *et al*., 1983). Also, classical PCR primers specific to sequences within the PSP-specific phaseolotoxin gene were available (Prosen *et al*., 1993; Schaad *et al*., 1995) for designing real-time PCR primers and probes. To further increase the sensitivity of PCR, membrane filtration has been combined with BIO-PCR in a high throughput format for ultra sensitive detection of bacteria in samples of water and leaf, and seed washings. Both classical nested PCR and a real-time PCR-based fluorescent 5′ nuclease assay system (Schoen *et al*., 1996; Schaad *et al*., 1997, 1999) were compared for detection of amplified products.

Materials and methods

Bacterial strains and pathogenicity tests

Bacteria used in this study and their sources are listed in Table 1. All PSP strains were tested for pathogenicity using bean (*Phaseolus vulgaris* cv. Hystyle) plants as described below, except the plants were at the 3–4 leaf stage and symptoms were recorded after 10 days.

Preparation of natural inoculum

To obtain natural inoculum, bean plants with several immature pods (3–7 mm long) and flowers were atomized with a suspension containing approximately 1×10^6 mean cfu mL^{-1} of PSP strains FC-74 or FC-1 and incubated in a lighted dew chamber (Pervical model E-54U-DL) at 18°C nights and 24°C days (12 h) until characteristic water-soaked lesions began to appear after 7–10 d on several leaves and pods. The plants were then transferred to a lighted, walk-in growth chamber set at 20°C nights and 24°C days (12 h) and with intermittent (30 s every 2 h) misting for three weeks. After an additional 30–32 d without misting, all watering ceased and the resulting mature pods with lesions were allowed to dry on the plants. Pods with dark green greasy-appearing lesions were collected and stored at room temperature in small coin envelopes. The seeds were removed from the pods, and lesion tissue cut out with a scalpel, pooled, and ground to a fine powder with a pestle in a sterile mortar. The seeds and ground tissue were stored separately in a Petri dish at room temperature until needed.

Media

King's medium B (KB) agar (King *et al*., 1954) and semiselective MSP agar (Mohan & Schaad, 1987) were prepared as described. Because preliminary results showed that PSP grew more rapidly on filters placed over soft agar than those placed over standard solid agar, all media for membrane BIO-PCR were made with 1% agar.

Classical and real-time PCR primers and probe

All PCRs, regardless of the method, were performed directly without extracting DNA (direct PCR) unless stated otherwise. Classical PCR using external primers P 3·1 and P 5·1 and nested primers P 3·2 and P 5·2 resulted in the expected 0·5 kb product for classical PCR and 0·45 kb product for nested PCR (Schaad *et al*., 1995), respectively. PCR was performed in 25 μL using 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM of $MgCl_2$, 0·001% gelatin, 80 μM each of dATP, dCTP, dGTP, dTTP and 0·2 units of *Taq* DNA polymerase. For nested PCR, 1 μL of a 10-fold diluted product of the external PCR product was used as template. In each experiment a Southern blot using a 0·45 kb fragment of the 2·6 kb *Eco*RI segment was performed to confirm the identity of the nested PCR amplified product, as described by Schaad *et al*. (1995).

For real-time PCR, the following primers and probe were selected based upon specificity to *P. syringae* pv. *phaseolicola*:

Forward primer PsF-tox 188: 5′ GGGGTGGGACGTGTTAT 3′

Reverse primer PsR-tox 557: 3′ GGCTGGCAGATTGATGTTC 5′

Probe PsF-tox 286P: 5′ ACCATCCGAATGCCAGTAATGCC 3′

These real-time primers and probe were designed from sequences within the same 2·6 kb *Eco*R1 fragment within the *tox-argK* chromosomal cluster of the *des*-like open reading frame (Hatziloukas *et al*., 1995) of the PSP-specific phaseolotoxin gene (accession U27310) from which the above PSP classical primers were designed (Schaad *et al*., 1995). For real-time PCR (TaqMan®), the probe was labeled with two fluorescent dyes: a reporter dye

Table 1 Strains of *Pseudomonas syringae* pv. *phaseolicola* (PSP), other pathovars of *P. syringae*, species of *Pseudomonas*, and unknown saprophytic fluorescent pseudomonads isolated from bean seed used in this study and their reaction to PSP tox gene-specific real-time PCR primers and probe[a]

Strain[b]	Species/pathovar	Source[c]	Amplification[d]
FC-22, 30	*P. syringae* pv. *phaseolicola*	N.W. Schaad, ID, USA	+
FC-1 (C-199)	″	N.W. Schaad, ID, USA	+
FC-42 (132)	″	M. Ozakman, Turkey	+
FC-52 (PM-180)	″	ICPB	+
FC-74 (1281A)	″ race-1	J. Taylor, UK	+
FC-75 (882)	″ race-2	J. Taylor, UK	+
FC-76 (1301B)	″ race-3	J. Taylor, UK	+
FC-77 (1348B)	″ race-4	J. Taylor, UK	+
FC-78 (34120)	″ race-5	J. Taylor, UK	+
FC-79 (34569)	″ race-6	J. Taylor, UK	+
FC-80 (1449B)	″ race-7	J. Taylor, UK	+
FC-81 (2656A)	″ race-8	J. Taylor, UK	+
FC-82 (2709B)	″ race-9	J. Taylor, UK	+
993	″	G. Franc, WY, USA	+
FC-41	″ (non-fluorescent)	J. Taylor, Tanzania	–
FC99 (B3A)	pv. *syringae*	S. Hutchenson, MD, USA	–
FC-103 (301D)	pv. *syringae*	S. Hutchenson, MD, USA	–
FC-101 (SD 302)	pv. *syringae*	S. Hutchenson, MD, USA	–
FC-105 (PS 17)	pv. *syringae*	S. Hutchenson, MD, USA	–
FC-53 (PS 270)	pv. *syringae*	ICPB	–
FC-54 (PS 22)	pv. *tomato*	ICPB	–
FC-59 (JL1)	pv. *tomato*	Dhanbantari, Canada	–
FC-68 (LG-2)	pv. *glycinea*	ICPB	–
FC-55 (PT130)	pv. *tabaci*	ICPB	–
FC-58 (PT-5)	pv. *tabaci*	ICPB	–
FC-127 (12623)	*P. putida*	ATCC	–
FC-62 (PF-107)	*P. fluorescens*	ICPB	–
FC-120 (13525)	*P. fluorescens* biotype A	ATCC	–
FC-23	Saprophyte, bean seeds	K. Mohan, ID, USA	–
FC-24	Saprophyte, bean seeds	K. Mohan, ID, USA	–
FC-25	Saprophyte, bean seeds	K. Mohan, ID, USA	–
FC-26	Saprophyte, bean seeds	K. Mohan, ID, USA	–
FC-27	Saprophyte, bean seeds	K. Mohan, ID, USA	–
FC-28	Saprophyte, bean seeds	K. Mohan, ID, USA	–
FC-29	Saprophyte, bean seeds	K. Mohan, ID, USA	–

[a]Real-time PCR run for 40 cycles.
[b]Number in parenthesis is the original source from which the culture was obtained.
[c]ICPB, International Collection of Phytopathogenic Bacteria, USDA/ARS, Ft. Detrick, MD, USA; ATCC, American Type Culture Collection, Manassas, VA, USA.
[d]+, Fluorescence rose above baseline between 18 and 25 cycles; –, fluorescence failed to rise above baseline after 40 cycles.

(FAM) and a quencher dye (TAMRA) (Schaad *et al*., 1999). When the probe is left intact in the reaction mix the quencher dye inhibits the reporter dye and there is no emission of fluorescence. If the probe hybridizes with the target DNA, the 5′ nuclease activity of the *Taq* polymerase cleaves the probe and frees the reporter dye. The emission of fluorescence produced by the free reporter dye is proportional to the amount of amplified DNA, and monitored in real time by the ABI 7700 Sequence Detection System (Applied Biosystems). PCR was performed in 25 μL reactions containing reagents at the following concentrations: TaqMan buffer1X, $MgCl_2$ 3 mM, dNTPs 250 μM, primers 0·4 μM, probe 0·1 μM, AmpErase 0·5 U, AmpliTaq Gold 1 U, and template, 12·8 μL. The PCR profile was: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C followed by 1 min at 60°C. Results were recorded as a simple positive or as cycle threshold (Ct) values. The Ct value is defined as the PCR cycle number at which time the signal (fluorescence) of the probe raises above background; negative, no fluorescence observed.

Specificity of TaqMan real-time PCR primers and probe

To test for specificity of the newly designed primers and probe for the real-time PCR assay, cells from cultures growing overnight on KB agar plates were transferred to PCR tubes, containing 10 μL water, with a sterile tooth pick and tested by real-time PCR for 40 cycles, as described above. Sixteen strains of PSP were tested, including one of each of nine races and a non-fluorescent strain (Table 1). Strains of several closely related pathovars of *P. syringae* including *syringae*, *tomato*, *glycinea* and *tabaci*

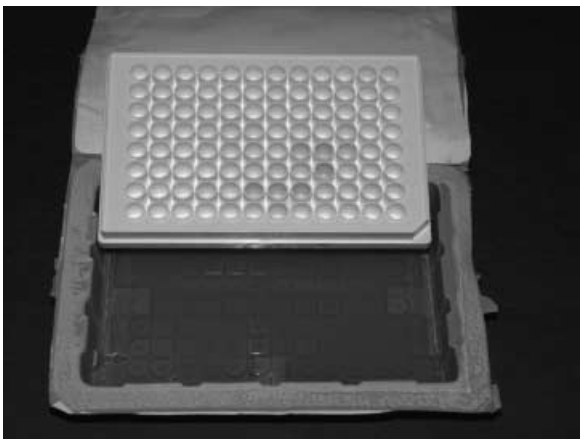

Figure 1 Membrane plate consisting of 96-wells removed from soft agar incubation chamber. Note marks of plate on the agar.

and distantly related pseudomonads *P. putida*, *P. fluorescens*, and seven unknown saprophytic bacteria recovered from bean seeds on KB agar were included (Table 1).

BIO-PCR

BIO-PCR was conducted as described (Schaad *et al*., 1995). Samples of 100 μL were spread onto each of eight KB and MSP agar plates and incubated at 28°C for 32–34 h and 48–50 h, respectively, to obtain pin point-size colonies of the target PSP organism. Results of dilution plating assays (using KB agar) of entire single colonies removed from MSP agar after 48 h at 23°C showed an average of 30 000 total cells present. Depending upon the experiment, each of five plates of each medium were washed three times with 1 mL of water and the washings pooled into a single sample of 15 mL or each plate was washed twice with 1 mL of water and the washings of each plate kept separate. The samples were kept on ice and 2·8 μL used immediately for direct PCR (without extraction of DNA) or stored at –20°C. The remaining three plates were incubated for an additional 24–48 h for determining mean cfu mL^{-1} and/or recovery of PSP.

Membrane BIO-PCR

A special 96-well micro titre plate (Fig. 1) fitted with surfactant-free 0·45 μ pore size cellulose nitrate filter (Sartorius Corp.) was designed to allow the membrane to be flush with the bottom of the plate and allow complete contact with the agar medium. The plates were made by special order by Corning-Costar and provided individually in a sterile package. Surfactant-free membranes were needed to prevent killing small numbers of bacteria by the surfactant (unpublished data). DNA from 1–2 dead cells cannot be detected by standard PCR but 1–2 viable cells can be detected by BIO-PCR protocols. Aliquots of 400 μL of each sample were dispensed into triplicate wells of each of two plates. The plates were then placed on a 96 well vacuum suction device (Whatman Polyfiltronics) to remove the liquid and the same operation repeated after adding two additional 400 μL aliquots so that 1·2 mL of the sample was filtered through each well. Separately, 100 μL of each dilution were spread onto each of five plates of KB and MSP agar plates and incubated under the same conditions to determine mean cfu mL^{-1} of each sample. After filtering the samples, one of the 96 well plates was placed onto KB or MSP soft agar in the original sterile plastic container (Fig. 1), slightly tapped to allow contact with the agar, and the lid added to keep the plate sterile. Alternately the plates were placed onto soft agar in a 20 × 40 cm Pyrex baking dish and covered with aluminum foil. After 32–34 and 48–52 h for KB and MSP, respectively, each well of the micro titre plate was washed with 200 μL of sterile water using a multi-channel micro pipette and the sample transferred to a microfuge tube for PCR or archived at –20°C. The second 96 well plate was used for membrane PCR without incubating on agar media. Each well was washed and 12·8 μL used for classical real-time PCR, as above.

Determination of sensitivity

Pure cultures in water

To evaluate the membrane technique for detection of PSP in water, an overnight culture growing in liquid NBY was adjusted to 0·1 OD at 600 nm and 10-fold serial dilutions made to 10^{-10} to obtain samples with one or fewer cfu per 100 μL. To determine actual number of cfu of PSP present, samples of 10^{-5}, 10^{-6}, and 10^{-8} dilutions were kept on ice and 100 μL aliquots of each plated onto each of three plates of KB agar. After incubating at 23°C for 55–60 h, the numbers of cfu were determined. Dilutions were stored at –20°C for PCR later. All samples were assayed by classical PCR, BIO-PCR, and membrane BIO-PCR using 12·8 μL of template. Both classical and real-time PCR were used for detection of PSP. For classical PCR, nested primers and a Southern blot were performed, as described above.

Seeds spiked with natural inoculum

For recovery of PSP from bean seed (cv. Hystyle), samples of PSP-spiked bean seed washes were used. Seed wash samples were spiked with natural PSP-inoculum obtained from dried lesions of infected pods, as described above. For sensitivity tests, a sample of 2·8 mg of the finely ground pod material was suspended in 2 mL of sterile water and kept at 4°C for 30 min. The seed pod suspension was then diluted in sterile water to 10^{-3}, and aliquots of 100 μL of each 10-fold dilution spread onto each of three KB agar plates and incubated at 23°C to determine mean cfu mg^{-1} tissue. After determining the approximate mean cfu mg^{-1} tissue, another sample was weighed, suspended in 2 mL water, incubated for 30 min, and 100 μL of the undiluted and each of the 10-fold dilutions used to spike four samples of 250 seeds in 250 mL of cold sterile water. Non-spiked samples of 250 seeds in 250 mL of water served as a negative control. All samples were incubated at 4°C for 17 h as described by Mohan & Schaad (1987). From each

sample, 100 μL aliquots of seed soak fluid were plated onto each of 20 plates of MSP. In a preliminary experiment with seed washings, samples for BIO-PCR were incubated on both KB and MSP agars. However, since BIO-PCR results were more consistent using the semi-selective MSP agar, MSP was used for BIO-PCR and KB was used for control colony counts. For BIO-PCR, after 48–52 h of incubation at 23°C, five plates were washed three times each with 1 mL of sterile water and then pooled for PCR. The other 15 plates were incubated for 72 h at 23°C to determine mean cfu mL^{-1}. For membrane BIO-PCR, three times 400 μL of each sample of seed soak fluid (1·2 mL total) were filtered into triplicate wells of each of two 96-well plates, as described above. After incubating for 48–52 h on MSP soft agar, each well of the micro titre plate was washed with 200 μL of sterile water as above and used immediately for classical and real-time PCR or stored at –20°C.

Leaves

To evaluate the detection of PSP from plant leaf surfaces, the recovery of the bacterium from bean leaves was tested using real-time PCR, BIO-PCR and membrane BIO-PCR. To obtain leaf samples with PSP, six bean plants at the 2–3 leaf-stage, for each treatment, were sprayed with a dilution of 10^{-4}, 10^{-5} and 10^{-6} of a pure culture of PSP prepared as above for recovery from water. Six 100 μL aliquots of each dilution were plated onto KB agar plates and incubated for 48 h at 23°C to determine the mean cfu mL present in the inoculum used to spray the leaves. After 2–3 h, when the leaves were dry, one leaf from each of six plants of each treatment (different dilutions) was collected in a quart size 'Zip Lock'™ plastic bag containing 50 mL of washing buffer (0·1 M KH_2PO_4, 0·1% Bacto peptone, 0·1% Tween 20, pH 7) and sonicated (low power) for 10 min. One millilitre aliquots of sonicated leaf washings were kept at –20°C for real-time PCR. For BIO-PCR, 100 μL aliquots of sonicates were plated onto each of eight MSP agar plates and incubated at 23°C. For each of the six leaf samples, five of the plates of MSP were washed after 50–52 h, respectively and assayed by real-time PCR. The three other plates (controls) were incubated for 3 days to determine the mean cfu mL^{-1} present in the leaf washing. For membrane BIO-PCR, 1 mL of sonicate from each of the six samples was filtered on each of three wells of two 96-well membrane plates and one plate incubated on soft MSP agar for 2 days and the other for 3 days, as above. Each well was then washed with 100 μL of sterile water and the washings stored at –20°C. For PCR, the six membrane washings were diluted to10^{-2} and 12·8 μL assayed by real-time PCR, as above.

Results

Pathogenicity

All strains of PSP, including non-fluorescent FC-41, produced water-soaked lesions surrounded by a yellow halo after 7–10 days.

Table 2 Comparison of sensitivity of detection of *Pseudomonas syringae* pv. *phaseolicola* (PSP) in spiked bean seed washings using direct PCR, BIO-PCR, and 96-well membrane BIO-PCR by classical PCR[a]

PSP, mean cfu mL^{-1}	Direct PCR[c]	Direct BIO-PCR[e]	Membrane BIO-PCR[f]
Negative Control[b]	0/2[d]	0/6	0/3
40	0/3	5/6	6/6
4·2	0/3	0/5	6/6
0·4	0/3	0/3	6/6

[a]Washings of samples of 250 bean seeds soaked in 250 mL water for 2 h were spiked with dilutions of PSP from powdered infected bean pods. Actual colony forming units per mL (cfu mL^{-1}) determined by spreading 100 μL onto each of five KB agar plates. All assays done using classical nested PCR with *tox* primers.
[b]Negative control consisted of soaking 250 seeds without adding PSP.
[c]Sample is 12·8 μL of seed washings used directly as PCR template.
[d]Number samples positive/number samples tested.
[e]Sample of 12·8 μL of plate washing obtained as follows: for each dilution 100 μL of seed washing were spread on each of five plates of MSP agar; the plates were incubated for 2 days and washed three times with 1 mL of sterile water; 12·8 μL of the pooled 15 mL were used as template in each PCR reaction.
[f]Sample of 12·8 μL of well washing obtained as follows: three times 400 μL of each of four seed washing samples (1·2 mL total) were filtered in triplicate wells of each of two membrane plates (six wells per dilution). After incubating for 2 days on soft MSP agar, each well was washed with 200 μL of sterile water and 12·8 μL of each of the well washings was used as template in each PCR reaction.

Specificity of real-time PCR primers and probe

Of the 16 strains of PSP tested, all were positive using the newly designed *tox* gene-specific real-time PCR primers and probe except strain FC 41, while all 13 other pseudomonads were negative, as were six unidentified saprophytic bacteria morphologically similar to PSP isolated from beans (Table 1).

Determination of sensitivity

Pure cultures in water

Membrane BIO-PCR resulted in a significant increase in sensitivity over BIO-PCR in detecting samples of pure cultures containing extremely small numbers of PSP. In the first series of experiments, with samples containing a mean of 17 cfu mL^{-1} (10^{-8} dilution) PSP was detected by BIO-PCR and membrane BIO-PCR but not by classical or membrane PCR using ethidium bromide stained gels and Southern analysis. With samples containing 12 or fewer cfu mL^{-1} (10^{-8} and 10^{-9} dilutions), membrane BIO-PCR detected 13 of 16 samples whereas BIO-PCR detected PSP in only 14 of 24 samples. In a later experiment with samples containing a mean of 12 cfu mL^{-1}, PSP was detected by membrane BIO-PCR using both real-time and classical detection techniques.

Seeds spiked with natural inoculum

Membrane BIO-PCR (96-well plate) combined with nested PCR and Southern blot analysis (Tables 2 & 3) or

Table 3 Comparison between the sensitivity of direct PCR, BIO-PCR, and membrane BO-PCR for detection of *Pseudomonas syringae* pv. *phaseolicola* (PSP) in spiked washings of bean seeds by classical nested and real-time PCR[a]

	Direct PCR[c,d,e]		BIO-PCR[f]		Membrane BIO-PCR[g]	
Treatment	Real-time	Classical nested	Real-time	Classical nested	Real-time	Classical nested
Negative control[b]	–	–[e]	–	–	–	–
910 cfu/ml^{-1}	–	+	+	+	+	+
26 cfu/ml^{-1}	–	–	–	+	+	+
2·0 cfu/ml^{-1}	–	–	–	–	+	+
<1·0 cfu/ml^{-1}	–	–	–	–	–	+

[a]Washings of samples of 250 bean seeds soaked in 250 mL water for 2 h were spiked with dilutions of PSP prepared from powdered infected bean pods. Actual colony forming units per mL (cfu mL^{-1}) determined by spreading 100 μL onto each of five KB agar plates.
[b]Negative controls consisted of soaked seed without adding PSP.
[c]+, Fluorescence rose above background within 40 cycles; –, fluorescence failed to rise above background within 40 cycles.
[d]Sample of 12·8 μL of seed washings used directly as PCR template without extracting DNA.
[e]+, 0·45 kb DNA band observed; –, no DNA band observed.
[f]Sample of 12·8 μL of agar plate washing obtained as follows: for each dilution 100 μL of seed washing were spread on each of five plates of MSP, the plates were incubated for 2 days and washed three times with 1 mL of sterile water, 12·8 μL of the 15 mL pooled sample were used as template in each PCR reaction.
[g]Sample of 12·8 μL of microplate well washing obtained as follows: three times 400 μL of each seed washings (1·2 mL total) were filtered in a well of the membrane plate (six wells per dilution), the plate was then incubated for 2 days on soft MSP agar and each well washed with 200 μL of sterile water and 12·8 μL of the well washing was used directly as PCR template without extracting DNA.

real-time PCR protocols (Table 3) were able to detect samples containing a mean of 4·2 cfu mL^{-1} or less. All other methods were negative at such low concentrations.

Detection from leaves

The mean concentration of inoculum present in the three leaf washing treatments, 10^{-4}, 10^{-5}, and 10^{-6}, was 5500, 500, and 55 cfu mL^{-1}, respectively. The inoculum recovered in the leaf washings for each respective treatment was 80, 40, and zero mean cfu mL^{-1}, respectively. The classical PCR and BIO-PCR assays were negative for PSP for all three treatments. In contrast, for membrane real-time BIO-PCR three out of three samples for treatment 1 (80 mean cfu mL^{-1}) were positive for PSP after 2 days of incubation with Ct values of 27·4, 29·8, and 33·1. After 3 days of incubation, Ct vales were 28·5, 29·7, and 29·7. For treatment 2 (40 mean cfu mL^{-1}), one of three was positive after 2 and 3 days with a Ct values of 34·4 and 31·3, respectively. For the third treatment (zero mean cfu mL^{-1}) all samples were negative after both 2 and 3 days incubation.

Discussion

Pseudomonas syringae pv. *phaseolicola* is a highly regulated seed-borne pathogen (Grogan & Kendrick, 1967; Webster *et al*., 1983). Several techniques for detecting PSP in seeds are available, including isolation on semi-selective agar media (Mohan & Schaad, 1987), serology (van Vuurde & van den Bovenkamp, 1989), and PCR (Prosen *et al*., 1993; Audy *et al*., 1995; Schaad *et al*., 1995). Membrane BIO-PCR using classical nested PCR or real-time PCR worked as well for ultra sensitive detection of PSP in seed washings as in pure culture in water. Use of bean seed washings containing known numbers of PSP showed the greatest sensitivity when the 96 well membrane BIO-PCR technique was combined with classical nested PCR and a Southern blot analysis. A detection limit of 26 mean cfu mL^{-1} by BIO-PCR is in agreement with the sensitivity originally reported for BIO-PCR to detect PSP in commercial seed lots (Schaad *et al*., 1995). Also, the results agreed with previous preliminary results showing the detection of 1–3 cfu of PSP per millilitre of pure culture using membrane BIO-PCR when combined with nested PCR and Southern blot analysis (Schaad *et al*., 1996).

If such ultra sensitivity is not needed, the more rapid and less costly real-time PCR technique using multi-well plates and the high throughput ABI 7700 Sequence Detection System is the method of choice. As expected, the target pathogen was not detected at concentrations of 2 mean cfu mL^{-1} by standard BIO-PCR (no membrane) even with nested primers and a Southern blot. However, PSP was detected at 2 mean cfu mL^{-1} with membrane BIO-PCR regardless of the detection method used to detect the amplified product. To detect such small numbers of cells it is necessary to use surfactant-free membranes because small numbers of cells are killed by the surfactant (N.W. Schaad, unpublished data). Such sensitivity is a 10-fold increase over the detection obtained by BIO-PCR and nested primers and nearly a 500-fold increase over BIO-PCR and real-time PCR. When large numbers of samples are to be assayed, the 96-well plate allows two replications of 40–45 samples, including controls.

Real-time PCR has many advantages over classical nested PCR, including speed and freedom from contamination (Schaad & Frederick, 2002). The higher cost of a fluorescent-based detection system is more than offset by the significant savings in labour costs resulting from the additional 6–8 h needed to do the classical assay. Real-time PCR has other advantages including being a closed

system and providing quantifiable results (Schaad & Frederick, 2002). A disadvantage of the real-time PCR system was the need to dilute the natural bean seed washes 10-fold or more; cleavage of the amplified product was apparently inhibited by plant materials in the seed wash. This thereby prevented fluorescence from occurring and certainly contributed to the lower sensitivity observed with real-time PCR over classical nested PCR. Both the PSP-specific *tox-argK* gene cluster (Hatziloukas *et al*., 1995) classical primers (Schaad *et al*., 1995) and the newly designed real-time primers and probe are highly specific but have a disadvantage in sensitivity since the target sequence exists as a single copy. The real-time primers and probe maintained the specificity of the classical primers (Schaad *et al*., 1995). However, the failure of the non-fluorescent strain to react suggests the primers and probe may be too specific. Also, a clonal group of nontoxigenic (lack *argK* gene cluster) strains of PSP has recently been reported in Spain (Rivas *et al*., 2005; Rico *et al*., 2003). Although nontoxigenic and nonfluorescent strains are not commonly observed (Schaad *et al*., 1995) and not generally available in international culture collections, such strains may occur in small numbers in field samples and simply not be recognized. This suggests that a multiplex PCR assay including primers and probe to the common *Tox* positive strains and less common *Tox*- and nonfluorescent strains may be needed.

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